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The *Drosophila* gene *wingless*, and its vertebrate homologue the proto-oncogene *Wnt-1*, encode extracellular signalling molecules that regulate differentiation and cell proliferation. During year one of our grant, we have made significant progress towards understanding the biochemical mechanisms by which the *wingless* signal is transmitted from cell to cell. Our work has shown that *wingless* protein (WG) is post-translationally modified by the addition of an N-linked glycosylation group. Once secreted, most of the extracellular WG is actually tethered to the cell surface and extracellular matrix. Several lines of evidence suggest that this association may occur via interactions with cell surface heparan sulfate molecules. Furthermore, interactions with these heparan sulfate groups can modulate the activity of the WG signal.

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INTRODUCTION:

Background:

Communication between individual cells and groups of cells is an integral part of development and differentiation. Signals from neighboring cells often activate key cell fate decisions. In many instances, the same signalling molecules that affect cell lineage differentiation and pattern formation also control cell proliferation. Two particularly intriguing examples of this are the proteins encoded by the *Drosophila* gene *wingless* (*wg*) and its mammalian homologue *Wnt-1* (1,2). These proteins share 64% sequence identity and may be functionally equivalent (3). Both contain a hydrophobic signal peptide; multiple, conserved cysteine residues; N-linked glycosylation site(s); and no discernible transmembrane domain (3). Null mutations in *wg* and *Wnt-1* are embryonic lethal, causing severe congenital malformation of the developing epidermis and central nervous system (4,5). *wg* and *Wnt-1* also regulate cell growth in certain cell types. *wg* is required for normal cell proliferation in the Malpighian tubule anlage (6), the developing wing discs (7), and neuroblasts (8); while ectopic expression of *Wnt-1* can stimulate cell division in the CNS (9) and induce growth of mammary tumors in adult mice (2).

It has been postulated that *wg* and *Wnt-1* are extracellular signaling proteins which mediate intercellular communication, thereby regulating differentiation. However, very little is known about the biochemical nature of this cell-cell signalling. That is, how is the ligand transmitted? How is it received? And why are different cells able to respond in a position-specific manner?

Other genes in the *wg / Wnt-1* pathway. Several other genes in the pathway have been identified genetically (for recent review see 10-12). A working model of how these genes interact is shown in Figure 1. Much of the *wg* signal transduction pathway is highly conserved throughout evolution. *armadillo* (*arm*) , *disheveled* (*dsh*), and *zeste white(3)* (zw(3)) encode proteins which have known mammalian homologues. *arm* is the best characterized of the three; it encodes the *Drosophila* homologue of the vertebrate protein B-catenin. Previous biochemical studies in vertebrate cell lines have shown that B-catenin is associated with E-cadherin in adherins junctions (13). Recently, two labs have found that *Wnt-1* activity can modulate the subcellular localization of B-catenin (14) and plakoglobin (15) thereby regulating cell adhesion. *wg* may also regulate the subcellular localization of *arm* protein (ARM) (16). Whether or not *wg* modulates cell adhesion is not known.

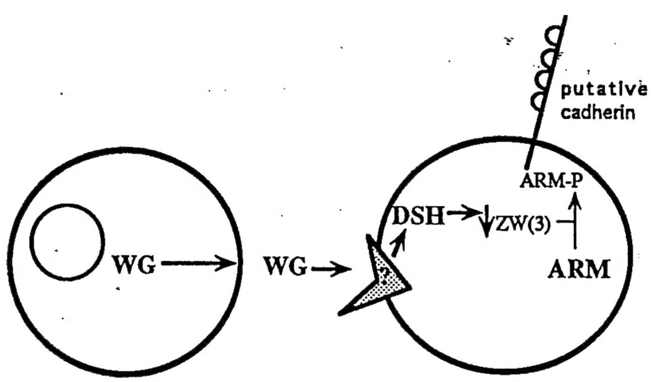

Figure 1. The *wg /Wnt-1* Signal Transduction Pathway.
The cell on the left expresses and secretes WG. *dsh* is the first gene known to act after reception of the WG signal. DSH represses ZW(3) kinase activity and there is a concomitant increase in the amount of unphosphorylation ARM protein present.

The extant genes in the pathway all encode cytoplasmic proteins. The steps required for transmitting the signal from cell to cell and receiving the signal are uncharacterized. One missing player in this scheme is of course the *wg* receptor. Classical genetic screens for zygotic segment polarity mutations have not identified candidate receptor(s).

Purpose of Present Work:

Genetic and molecular studies have shown that *wg* and its murine counterpart, the proto-oncogene *Wnt-1*, encode secreted proteins. These proteins are thought to act as extracellular signals that control growth and cell fate decisions in neighboring cells. Our goal is to understand how the signal is transmitted from cell to cell, and how the information is transduced in the receiving cells. The work outlined in this grant has divided into three sections:

First: Secreted WG protein regulates the expression, and perhaps the cytoplasmic location of ARM. We will define when during embryogenesis *wg* activity is needed for proper *armadillo* expression. Specifically, is *wg* required for maintenance as well as initiation of ARM accumulation? **Second**: We have constructed a tissue culture line which secretes soluble, active WG protein. WG activity can be followed using a second responding cell line, clone-8 cells. When clone-8 cells are grown in the presence of active WG protein, they express high levels of ARM protein. With both a source of soluble WG protein, and an assay for activity, we will purify WG protein, and determine if WG alone constitutes the signal or if other components are also required. **Third**: Identify and analyze accessory proteins that associate specifically with WG on the surface of the signalling cells and the responding cells, including candidate receptor molecules.

BODY / PROGRESS:

Specific Aim 2 Purification of WG Protein:

Our goal is to understand the extracellular steps in the *wg* / *Wnt-1* signalling pathway. In the past, biochemical analyses of *wg* / *Wnt-1* function have lagged behind genetic studies due primarily to the lack of an in vitro assay and purified WG protein. Recently, two in vitro assays have been developed for WG. In order to obtain WG protein in quantities sufficient for purification, we have constructed a genetically engineered cell line (S2hsWG(+)) which secretes active, affinity tagged WG (17). We have measured WG activity in both assays. In the first assay, S2hsWG(+) cells are co-cultured with specific populations of embryonic cells purified from viable Drosophila embryos by Whole Animal Cell Sorting (WACS) (17,18). The embryonic cells respond to WG expression by expressing specific downstream genes, such as *engrailed* (see Figure 3 in 17). In the second assay (19), clone-8 cells, which are an established cell line derived from wing discs, are treated with conditioned media from S2hsWG(+) cells. In response to the WG signal, the clone-8 cells express higher levels of unphosphorylated ARM (Figure 2a). Note that both the level of ARM expression, and the ratio of phosphorylated to unphosphorylated ARM is regulated by WG protein. We have found that concentrations of WG as low as 100 pM can elicit an ARM response (Figure 2b). This range of activity suggests an interaction with a high affinity receptor.

<u>Post-Translational Modification and Extracellular Localization of WG:</u>

Studies in our lab have shown that WG is post translationally modified by the addition of N-linked glycosylation groups. Treatment of S2hsWG(+) cells with tunicamycin effectively inhibits the gycosylation. WG undergoes similar post-translational modification in vivo. WG protein from whole cell extracts of S2hsWG(+) cells and of *P[hsWG]/TM3Sb* embryos show identical electrophoretic mobilities when examined by Western analysis.

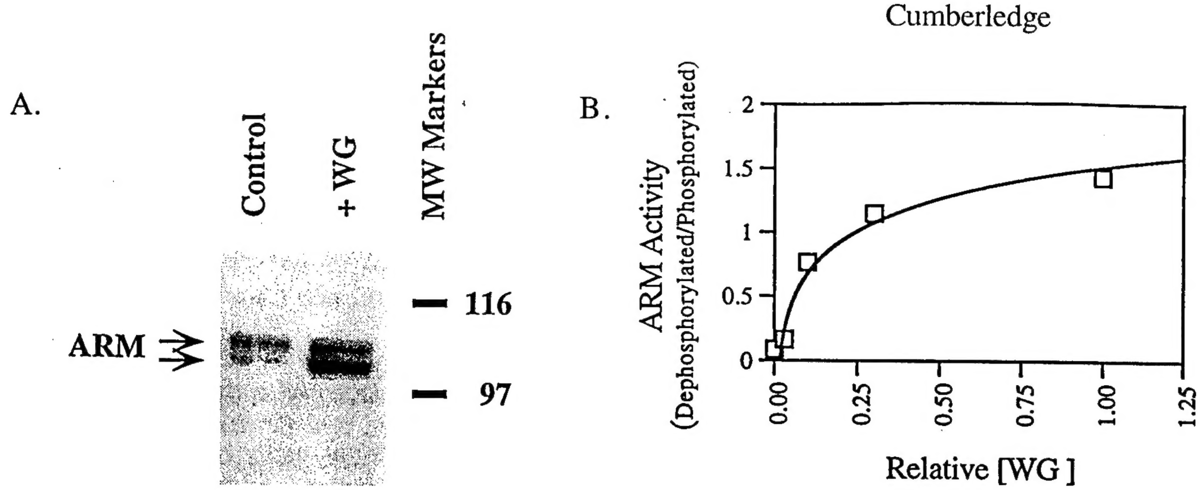

Figure 2. A. Assay for WG activity. Conditioned media from S2hsWG(+) and control S2hsWG(-) cells was applied to clone-8 cells. After 2 hours the clone-8 cells were harvested, lysed, and analyzed by SDS PAGE and immunoblotting with anti-ARM Mab. Equal amounts of protein were loaded in each lane. **B. Dose Response Curve**. ARM activity is expressed as the ratio of dephosphorylated ARM protein to phosphorylated ARM for each sample.

Once secreted, WG protein is partitioned into specific extracellular locations. We have metabolically labeled S2hsWG(+) cells with 35[S] methionine and used immunoprecipitation techniques to quantitate the amounts of WG found on the cell surface, extracellular matrix (ECM) and in the medium. Most of the secreted WG is tethered to the cell surface (41%) or ECM (40%); while little is found free in the medium (19%). We are now characterizing the nature of these cell surface interactions (see below).

Affinity chromatography using heparin agarose:

Using the clone-8 cell assay, we are purifying active, secreted WG from S2hsWG(+) cells. We have already evaluated several types of affinity chromatography reagents for use in purification of WG protein from conditioned media. WG binds with high affinity to the mannose-specific lectin Concanavalin A, but not to the fucose lectin LCA. This is in agreement with reports that while N-linked glycosylated proteins are found in insect cells, the high mannose groups do not typically undergo extensive processing to form "complex " sugar structures. Although WG binds to Concanavalin A-agarose, we have not been able to successfully elute active WG protein from the column, even with the addition of 1.5 M α-methyl-glucoside.

We have found that heparin agarose affinity chromatography is a far more effective initial purification step. Secreted WG protein binds to heparin agarose beads (see below for a discussion of WG interaction with cell surface heparin sulfate groups). This binding is high affinity; indeed, elution of WG required 1M NaCl. The majority of the extracellular heparin binding proteins are eluted at lower ionic strength. Based on our preliminary studies we estimate this purification step results in a 75% yield of WG protein, and approximately a 100 fold purification. In addition, this step provides a quick effective means to process large volumes of media, and to concentrate the WG protein.

Specific Aim 3 Identification of cell surface accessory proteins and candidate receptor molecules:

As an off shoot of the protein purification work, we have become interested in how WG protein interacts with the cell surface. Our recent work (see below) suggests that WG protein

may interact with surface heparin sulfate groups. This is an intriguing idea given that many growth factors (e.g. the FGF family;) are known to associate with cell surface heparin sulfate proteoglycans (HSPGs). This association is thought to play a significant functional role in the signal transduction process. For example, bFGF binding to syndecan, a HSPG, promotes FGF stability, and binding to the FGF receptor (20, 21). Although referred to as low affinity binding, FGF binds to syndecan with an affinity of 10^{-9}M.

We have found two lines of evidence suggesting that WG may also bind to a cell surface HSPG, and that this association may have a functional role in signal transduction. WG is released from the cell surface by the addition of exogenous heparin sulfate. Characterization of the S2hsWG(+) cells shows that most of the extracellular WG protein (>80%) is not freely diffusible in the media, but rather is tightly bound to the cell surface and ECM (our unpublished results (22). This association with the cell surface is non-covalent . Treatment with 0.1 % triton or .5M NaCl will not release WG. However, additional of as little as 10 μg/ml heparin sulfate is suff i cient to release most of the WG from the cell surface and ECM, suggesting that soluble heparin sulfate can effectively compete with sites on the cell surface. Cell surface associated WG can also be released by treatment with heparinase. Finally, as discussed above, WG also binds tightly to heparin agarose in vitro. Together, these results argue that WG may interact directly to heparin sulfate moieties on the cell surface.

Other experiments carried out in the lab indicate that cell surface heparan sulfate groups can play a functional role in the *wg* signal transduction pathway. We have observed that WG activity can be stimulated by the presence of exogenous heparin. Addition of 10 *ug*/heparin to the conditioned during the clone-8 paracrine assay stimulates WG activity about 4 fold. Conversely, when clone-8 cells are grown in the presence of 1 mM chlorate, thereby replacing cell surface sulfate groups, WG activity is reduced three fold. Addition of 10 μg/ml heparin sulfate to the chlorate treated cells during the incubation with WG can overcome this inhibition. These results also support the hypothesis that extracellular sulfated proteoglycans may play a functional role in localization of WG and transmission of the signal from cell to cell.

CONCLUSIONS

During year 1 of the grant we have made significant progress in two of our specific aims. The studies carried out this year have yielded important insights into the post-translational modification and extracellular localization of WG. In addition, we have made progress in understanding how WG protein interacts with the cell surface. We are currently writing a manuscript describing some of these results. Using information from these studies we have also been able to chose experimental techniques which will be useful for protein purification. For example, we were able to use heparin sulfate affinity chromatography to partially purify and concentrate WG protein. In the coming year, we will focus primarily on the protein purification. We anticipate that the next steps will involve fractionation using HPLC. In addition, we are testing the effects of glycosylation on WG activity. Finally, we are beginning chemical cross linking studies designed to identify cell surface proteins which interact with WG.

These studies are helping to create a picture of how the WG/Wnt-1 signal is transmitted. Understanding the extracellular events involved in signal transduction is an integral part of understanding the etiology of cancer. Because these events are extracellular they are particularly attractive candidates for future work focusing on modification of the signalling response, and treatment of breast cancer.

Modification of the Timeline for the Statement of Work:

In the original statement of work, we proposed that months 1-18 would be devoted primarily to characterizing *wg* dependent activation and maintenance of *armadillo* during germ band elongation and early contraction. According to this time line, our later studies would focus on purification of WG protein (months 10 -28) and the identification of factors that associate with WG on the surface of S2hsWG(+) (months 24-26).

Our preliminary work on the protein purification, and our studies on the role of cell surface sulfated proteoglycans have produced significant results sooner than we had anticipated. Therefore we have chosen to contine with these lines of investigation and to postpone characterization of the ARM response in vivo until months 20-38.